Jour. agric. Res. China (中華農業研究) 30 (1) : 5～13 (1981)

Effects of nitrogen supply to donor plants on pollen embryogenesis in cultured tobacco anthers[1]

Hsin Sheng Tsay[2]

ABSTACT : Levels of nitrogen supplied to culture solution had definite effects on the development and physiological aspects of tobacco plants (*Nicotiana tabacum* L. cv. Wisconsin 38). The correlation between nutritional status of the source plants and embryogenic characteristics of the excised anthers cultured *in vitro*, as well as the related histological explanations were demonstrated. Based on Hoagland's basic salts, 15 mM of nitrogen supplied to culture solution was most optimal for plant growth among the five nitrogen levels (0, 1.5, 4.5, 15.0, and 45.0 mM) tested. The highest embryogenic ability of cultured anthers was obtained when donor plants were subjected to 15 mM nitrogen treatment. Low nitrogen supply lowered the embryogenic ability. Histological study on microsporogenesis *in vivo* revealed that anthers obtained from the O nitrogen treatment had poorly developed tapetum layer and contained microspores with lightly stained cytoplasm. It was proposed that a better-developed tapetum layer and microspores with densely stained cytoplasm were important for haploid embryogenesis in tabacco anther culture.

Haploid plants are of special interest to geneticist and plant breeder because by chromosome doubling completely homozygous diploid plants can be obtained. Guha and Maheshwari (1964, 1966) demonstrated that immature anthers of *Datura innoxia*, when cultured in an appropriate nutrient medium, could give rise to plants with a haploid number of chromosomes [6,7]. This opened up a new approach to the induction of haploidy in plants. Soon it was found that this technique could be effectively employed in other crops, e.g., *Nicotiana*, *Oryza*, *Brassica*, *Hordeum* and *Triticum*, etc. Successful anther culture with haploid plant production has now been reported in 70 species, belonging to 37 genera and 12 families [17].

There are many factors which can influence the embryogenic ability of pollen in cultured anthers. One of the most important factors is the quality of the donor plants. Nitrogen supply usually plays an important role in maintaining plants in a vigorous and healthy condition. It is possible that it may also influence the haploid embryogenesis. The objectives of this research were to determine the relationship between nitrogen supply to the donor plants and pollen embryogenesis in cultured tobacco anthers and to

1. Contribution No. 968 from Taiwan Agricultural Research Institute.

2. Senior Agronomist, Department of Agronomy. TARI. Wufeng, Taichung Hsien, Taiwan 431, ROC.

explore the cytohistological basis for this relationship.

Materials and methods

Plants of *N. Tabacum* L. cv. Wisconsin 38 were raised from seeds sown in 1 : 1 Sponge rock and Canadian Sphagnum and covered lightly with quartz sands. When the plants were about 4 inches in height with 11 leaves, the roots were washed carefully and the plants were transplanted into culture solution, using 3-gal plastic containers covered with a wooden cover to reduce evaporation and to support the plants. Air was pumped into the culture solution continuously. The plants were grown in a greenhouse with the roof spreaded with heavy white paint to lower down the temperature in the summer months. Light intensity in the greenhouse was about 30,000 lux as measured at noon time.

Hoagland basic salts [8] with modified Fe, Mn and Mo salts [18] were used for growing the tobacco plants. Varying nitrogen levels based on Hoagland basic salts were shown in Table 1. The pH value was adjusted to 5.0 when first prepared, and was checked and readjusted every week during the culture. The culture solution was renewed at nearly 3 weeks interval. Distilled water was added to the container daily to maintain the culture solution at fixed volume. Each treatment contained 5 plants. The experiments were carried out in the University of California, Riverside, California, USA, in 1977-1978.

Table 1. Nitrogen levels and modifications of Hoagland's solution for culture of tobacco plants

mM Nitrogen in culture solution	Salts	mM	mg/l
0.0	$CaCl_2 \cdot 2H_2O$	5.0	735
	KCl	5.0	375
1.5	$Ca(NO_3)_2 \cdot 4H_2O$	0.5	118
	KNO_3	0.5	50.5
	$CaCl_2 \cdot 2H_2O$	4.5	661.5
	KCl	4.5	337.5
4.5	KNO_3	4.5	454.5
	$CaCl_2 \cdot 2H_2O$	5.0	735
	KCl	0.5	37.5
1.5	$Ca(NO_3)_2 \cdot 4H_2O$	5.0	1,180
	KNO_3	5.0	505
45.0	$Ca(NO_3)_2 \cdot 4H_2O$	5.0	1,180
	KNO_3	5.0	505
	NH_4NO_3	15.0	1,200

Flower buds with the sepal and petal in about equal length were removed from the donor

plants. They were sterilized by 5% Purex for 10 minutes and washed several times in autoclaved distilled water. Anthers were aseptically removed from flower bud and inoculated in 150×25mm test tubes containing 25 ml of Murashige and Skoog (1962) mineral salts [13] supplemented with 0.4 mg/l thiamine·HCl, 100 mg/l inositol and 3% sucrose. After adjusting the pH value to 5.7±0.1, 0.8% Phytagar was added. The medium was autoclaved at 15 lb/in^2 for 15 minutes and used within one week. During excision of anthers special care was taken to ensure that the anthers were not injured. Damaged anthers were discarded as they often tended to produce callus from somatic tissues. Cultures were maintained in a culture room at 27°±1°C and under 16 hr daily illumination with 1,000 lux fluorecent light. Each flower bud contained 5 anthers, three anthers were cultured, one was fixed in FAA for histological study, one was freshly squashed in a drop of 1% aceto-carmine to determine the microspore developmental stage. Only the anthers with normal microspores and around the first pollen mitotic stage were cultured; those containing microspores at other stages or without microspore were discarded. The percent of embryogenic anthers and the number of plants regenerated per anther were recorded 7 weeks after culture as preliminary results indicated that they reached maximum at this stage of culture. Standard error or 5% confidence limits of binomial variation were calculated for statistical analysis.

Flower buds of various developmental stages from donor plants growing in culture solution with different levels or nitrogen were collected for histological study. After fixation, anthers were dehydrated in TBA series and embedded in paraplast. Sections were cut at 10 μm, stained in Heidenhain's iron hematoxylin, and examined under a light microscope.

Results

I. Growth and flowering of plants in relation to nitrogen supply in solution culture.

It was observed that the control treatment (15 mM nitrogen) was optimum for plant growth in all aspects, i. e., plant hight, stem diameter, number of leaves, fresh weight and root condition (data not shown). Nitrogen concentrations in culture solutions also influenced the days required to flowering; the lower the nitrogen level, the longer the duration between transplanting and flowering. However, there was no conspicuous difference among treatments with nitrogen concentrations above 4.5 mM. Flowering dates of tabacco plants grown in culture solutions with O and 1.5mM nitrogen were 3-5 months and 10-30 days, respectively, later than other treatments.

Both excess and deficient nitrogen treatments produced fewer flower buds per plant. The fewest number of flower buds occurred in the O mM treatment with only about 20 flower buds per plant in the 2-month flowering period, whereas the 15 mM nitrogen treatment produced over 100 flower buds in the same period.

II. Anther culture

The results in Table 2 showed that there was no significant difference in percent of embryogenic anthers among 1.5, 4.5, 15.0 and 45.0 mM nitrogen treatments. However, a lower percentage was recorded from the O mM treatment. The data also indicated that nitrogen deficiency in culture solution caused a decrease in number of regenerated

plants per embryogenic anthers. Anthers produced from culture solution containing no nitrogen had the poorest embryogenic ability.

Table 2. Anther culture of *N. tabacum* at different culture dates affected by five nitrogen concentrations applied to donor plants.

mM Nitrogen in culture solution	Number of anther cultured	Embryogenic anthers			Total plants produced	No. of plants per embryogenic anther
		No.	%	0.95 CL	$\bar{X}$	
October-17						
4.5	33	18	54.5	35-71	446	24.8±5.7
15.0	30	23	76.7	57-90	971	42.2±8.1
45.0	33	13	39.4	24-59	542	41.7±9.0
October-20						
4.5	36	23	69.3	52-84	238	10.4±2.2
15.0	33	24	72.7	54-87	1,362	56.8±13.8
45.0	33	22	66.7	48-82	1,255	57.1±11.2
October-27						
4.5	39	16	41.0	25-59	251	15.7±3.7
15.0	60	39	65.0	53-78	1,003	25.7±7.0
45.0	30	20	66.7	47-83	390	19.5±5.6
November-1						
4.5	30	23	76.7	57-90	1,223	53.2±12.2
15.0	21	9	42.9	23-67	524	58.2±15.2
45.0	42	26	61.9	46-77	807	31.0±7.2
November-7						
4.5	39	22	56.4	39-73	276	12.6±5.4
15.0	36	24	66.7	48-82	417	17.4±4.0
45.0	39	23	59.0	42-75	570	24.8±6.6
Combine all 5 dates cultured						
0.0	39	15	38.5	24-57	289	19.5±6.6
1.5	181	134	74.0	66-81	2,732	20.4±2.6
4.5	177	102	57.6	50-66	2,434	23.9±2.6
15.0	180	119	66.1	58-72	4,277	35.9±3.2
45.0	177	104	58.5	51-67	3,564	34.3±3.2

The relation between anther embryogenesis and changes in nitrogen supply to donor plants was shown chronologically in Fig. 1. Under low nitrogen (4.5 mM) condition depletion of nitrogen in culture solution with time resulted in decrease in number of plants regenerated per embryogenic anther, whereas renewal of culture solution resulted in significant increase in embryogenic ability. The results were reversed when plants were grown under high nitrogen conditions (45 mM). Data on O and 1.5 mM treatments were not shown in Fig. 1. because of different flowering dates.

III. Histological Examination

A. Microsporogenesis

a) Normal sequence of microspore development

On the cross-section of an immature anther the following layers of cells can be distinguished : one layer of epidermal cells, endothecium, 1-3 layers of middle layer, and one innermost layer of tapetum comprised of cells with dense cytoplasm (Fig. 2A). At the beginning of meiosis, the tapetal nuclei may also undergo some divisions thus form two or more nuclei which by the end of the second meiotic division may increase

to a dozen or more. Normally proceeded meiosis produces microspores of spherical or eliptical shape. Twenty-four bivalents at metaphase I were showed in Figs. 2A-B. Some cells of the tapetum divide and form a second layer which is distinctly visible at the tetrad and early uninucleate stage (Fig. 2B). Microspores at late uninucleate stage were stained darker and had bigger nucleus than those at early uninucleate stage (Fig. 2C). The tapetum began to degenerate during the period of first pollen mitosis (Fig. 2D). Under natural condition anthers have an almost completely degenerated tapetum by the time of reaching the binucleate pollen grain stage (Figs. 2E-F). In late binucleate stage, the microspores were fully packed with starch grains (Fig. 2F).

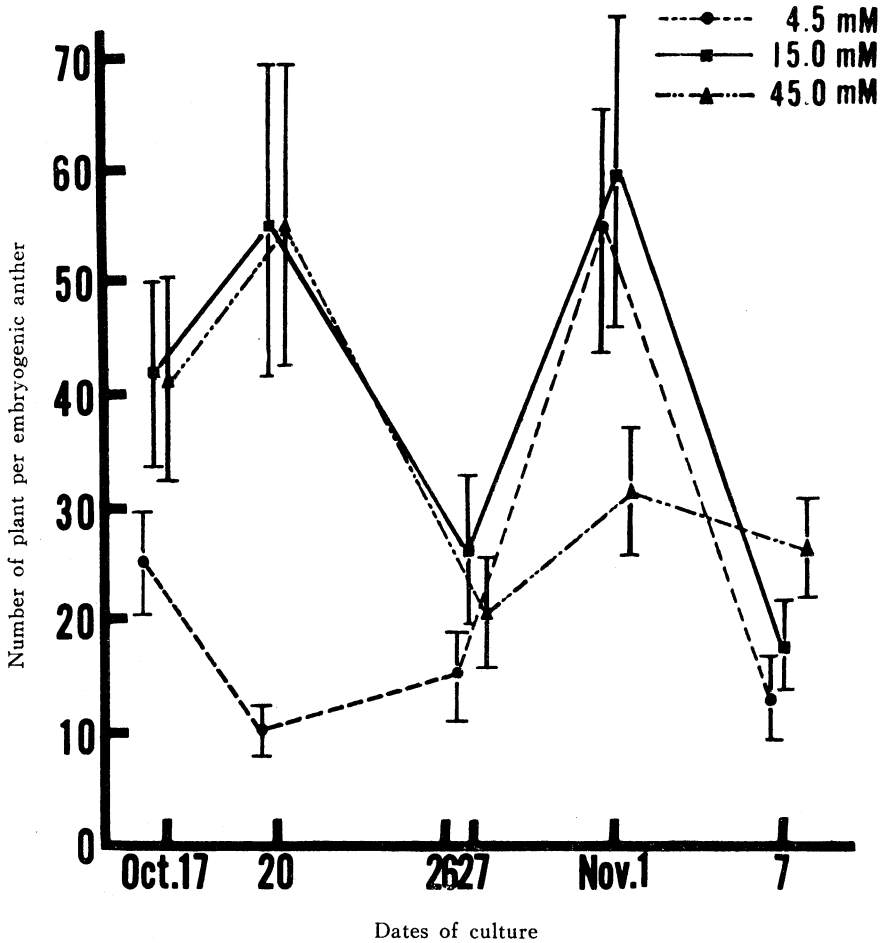

Fig. 1. The number of plants regenerated per anther at different culture date of three levels of nitrogen concentrations. (Culture solution renewed on October 26)

b) The tapetum differences and staining effect on cytoplasm of microspores among various nitrogen treatments.

Morphological differences of tapetum as a result of nitrogen treatment observed at first pollen mitosis stage were shown in Fig. 3. It was found that tapetum layer of tobacco plants grown in culture solution without any nitrogen had degenerated around

first pollen mitosis. The presence of tapetum layer was noted in all the other treatments. Comparing this phenomenon to the poor embryogenic ability of anthers from the O mM nitrogen treatment, the important role of tapetum in embryogenesis could thus be recognized.

Microspores showed densely stained cytoplasm in the 1.5, 4.5, 15.0, and 45.0 mM but not the O mM nitrogen treatments (Fig. 3). This was presumably resulted from the nutritive status of donor plants.

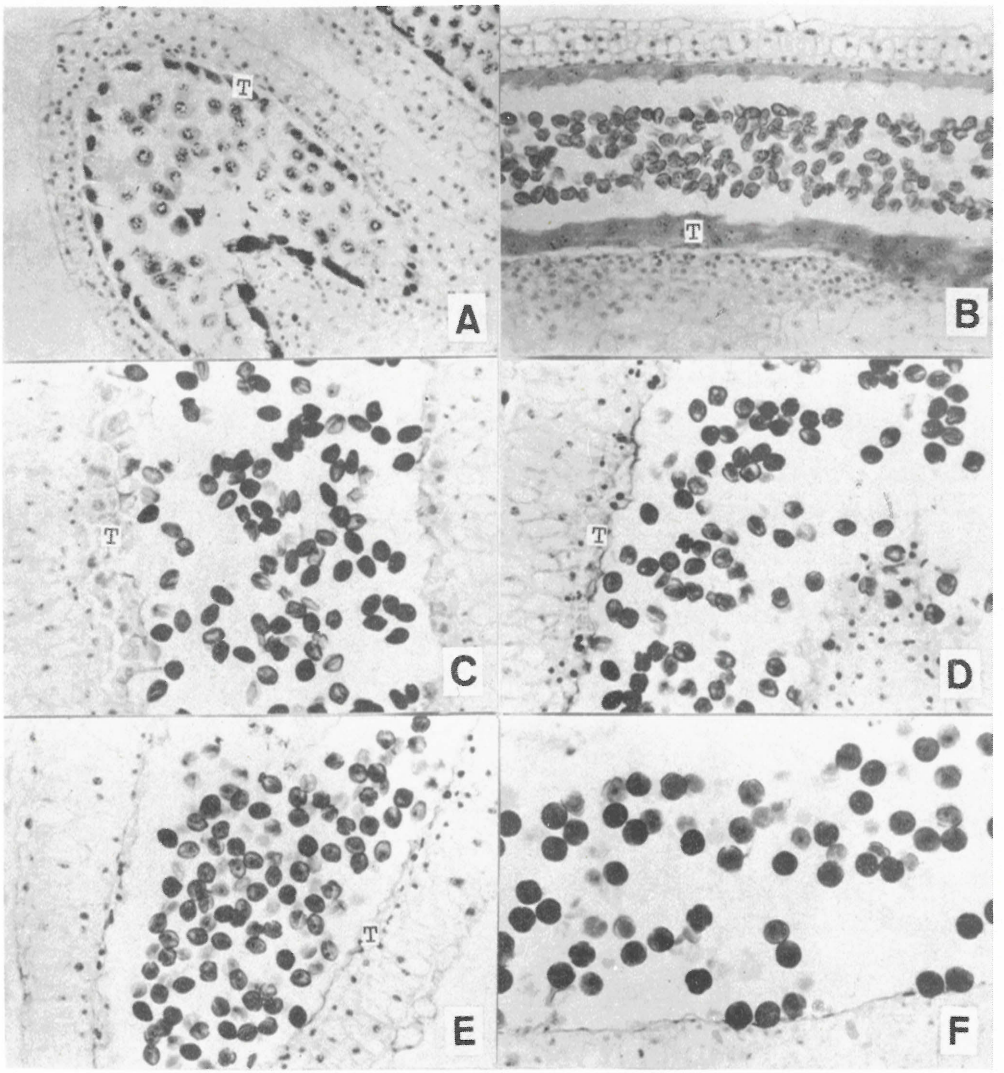

Fig. 2. Microspores of *N. tabacum* at different developmental stages. A : meiosis-diakinesis ; B : early uninucleate stage, microspores in foot ball shape, stained lightly ; C : late uninucleate stage, microspores stained darker and bigger than at early uninucleate stage ; D : first pollen mitosis stage ; E : early binucleate stage, starch grains not formed yet ; F : late binucleate stage, with starch grains. Magnification : 550X. T : tapetum.

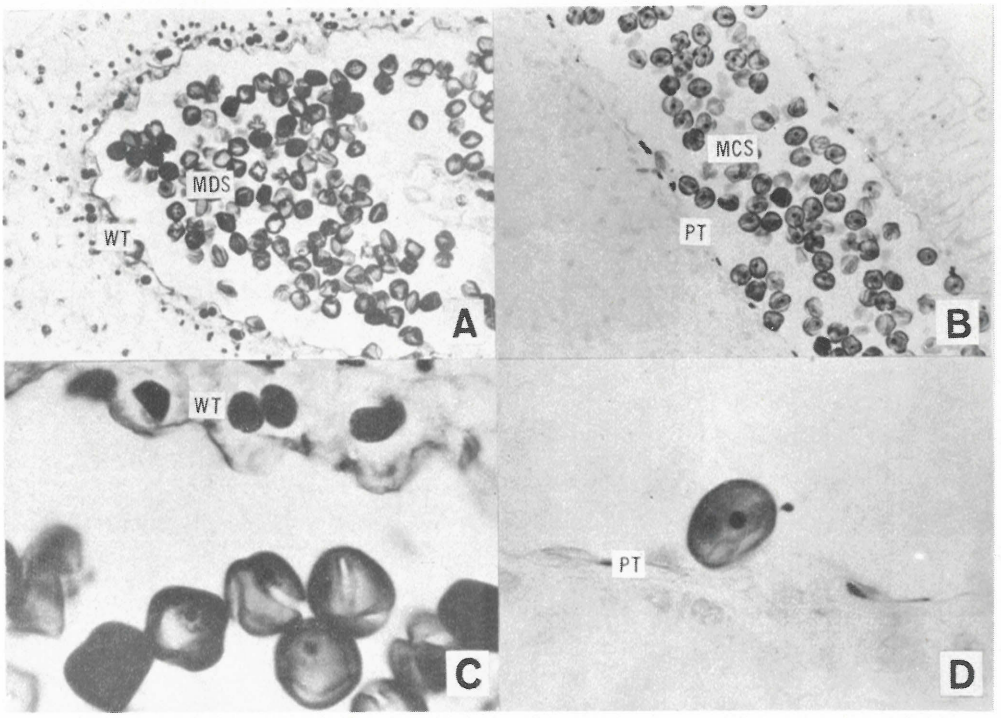

Fig. 3. The morphology of tapetum and staining effect on cytoplasm of microspores affected by nitrogen levels applied to donor plants. Nitrogen concentrations in culture solutions were 15 (Fig. A and C) and O (Fig. B and D) mM. Well-developed tapetum (WT) and microspores with densely stained cytoplasm (MDS) were observed in 15 mM nitrogen treatment, whereas O mM nitrogen treatment had poor developped tapetum (PT) and microspores with clearly stained cytoplasm (MCS). Magnification : A-B 550X, C-D 2250 X.

Discussion

The quality of donor plants is one of the most important factors which can affect the embryogenic ability of anthers in culture. For example, barley gave better results when grown out of doors than in the green house [2]. There were seasonal drifts in the frequency of haploid production in *Datura* [5]. Plant age could also cause significant differences in haploid production [3,14,16]. Light intensity and photoperiod under which the donor plants were grown were other factors influencing anther embryogenesis [3,4,9]. Considerable evidence has been accumulated to support the view that physiological condition of donor plants and environmental condition under which the plants were grown have played significant roles in haploid production.

Amount of nitrogen supplied to tobacco plants had profound effects on their development and physiological status. Among the five nitrogen levels tested in this study, the optimal concentration was 15 mM. All the data showed that nitrogen concentration in

culture solution of donor plants can strongly influence the embryogenic ability in anther culture. The reasons of lower embryogenic ability resulting from low nitrogen supply seem not due to nitrogen concentrations in anthers. (The results will be published in the seperate paper.) According to the histological study of microsporogenesis, it was clear that anthers obtained from 0 mM nitrogen treatment had the least developed tapetum layer and microspores containing lightly stained cytoplasm. When connecting this phenomenon to the poor embryogenic ability, it is apparent that tapetum layer and microspores with densely stained cytoplasm are of importance in anther embryogenesis. Tapetum layer has long been thought to have a nutritive function in *in vivo* microsporogenesis [11]. Moss and Heslop-Harrison (1967) found that more DNA is released by the tapetum than should be needed for the developing spores. Tapetum cells are useful because the synthesis of DNA is faster in polyploid than in diploid cells, and therefore can be used as reservoirs of DNA when a sudden demand occurs. This is perhaps what happens in the tapetum of pollen sacs when it breaks down and provides the reproducing cells with DNA and other substances for the rapid multiplication of essential cells [1]. However, few reports have mentioned the importance of tapetum layer in *in vitro* pollen embryogensis. Misiura and Zenkteler (1973) stated that tapetum layer in the form of a thin strip of cells was present in cultured anthers of *N. tabacum* [12]. Sunderland and Roberts (1977) suggested that the degenerating tapetum can probably provide nutrient to the embryogenic microspores [15]. Kohlenbach et al. (1978) demonstrated that in liquid anther culture of *N. tabacum* cv. Atropurea, the tapetum material is responsible for the androgenetic development of isolated microspores [10].

From the results of this study, it is obvious that the role of nitrogen supply is critical in haploid embryogenesis of tobacco. The influence of potassium, phosphorus and other elements should also be examined by using the same methods. These studies may also be expanded to see how pathogens, e. g., virus, affect plant health and consequently the success of anther culture.

Acknowledgements : I am especially grateful to Dr. Toshio Murashige for providing facitities and guidance during the period in which the research was carried out in Univ. of Calif. Riverside, USA. I also thank Dr. Dah-Jiang Liu for his kindness in reading the manuscript and making valuable suggestions.

Literature cited

1. Clowes, F. A. and B. E. Juniper. 1968. Botanical Monographs. Volume 8 : Plant cells. Blackwell Scientific Publications.
2. Dale, P. J. and M. W. Humphreys. 1973. Tissue culture. Rep. Welsh Plant Breeding Stat. No. 88.
3. Dunwell, J. M. 1976. A comparative study of environmental and developmental factors which influence embryo induction and growth in cultured anthers of *Nicotiana tabacum*. Environ. Exp. Bot. 16 : 109-119.
4. Dunwell, J. M. and M. E. Perry. 1973. The influence of *in vivo* growth conditions of *N. tabacum* plants on the *in vitro* embryogenic potential of their anthers. John Innes Ann. Rept. No. 64.
5. Engvild, K. C., I. Linde-Laursen and A. Lundquist. 1972. Anther cultures of *Datura innoxia* : flower

bud stage and embryoid level of ploidy. Hereditas 72 : 331-332.
6. Guha, S. and S. C. Maheshwari. 1964. *In vitro* production of embryos from anthers of *Datura*. Nature 204 : 497.
7. Guha, S. and S. C. Mashehwari. 1966, Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212 : 97-98.
8. Hoagland, D. R. and D. I. Arnon. 1938. The water culture method for growing plants without soil. Univ. of Calif. Agri. Exp. Sta. Circular No. 347 : 1-39.
9. Kasperbauer, M. J. and G. B. Collins. 1974. Anther-derived haploids in tabacco : Evaluation of procedures. Crop Sci. 14 : 305-307.
10. Kohlenbach, H. W., W. Wernicke and H. Lang. 1978. Androgenetic development of isolated microspores of *Hyoscyamus* and *Nicotiana*. 284-294. In: Production of natural compounds by cell culture methods. (Ed. by A. W. Alfermenn and E. Reinhard). Gesellschaft für Strahlen-und Umweltforschung mbH, München.
11. Maheshwari, P. 1950. An introduction to the embryology of angiosperms. 28-53. McGraw-Hill, New York-Toronto-London.
12. Misiura. E. and. M. Zenkteler. 1973. Studies on the androgenesis in cultured anthers of *Atropa belladonna* L. Acta Secietatis Betanicorum Poloniae. XLIIL 309-322.
13. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue culture. Physiol. Plant . 15 : 473-497.
14. Sunderland, N. 1971. Anther culture : A progress report. Sci. Prog. Oxf. 59 : 527-549.
15. Sunderland, N. and M. Roberts. 1977. New approach to pollen culture. Nature 207 : 236-238.
16. Tomes, D. T. and G. B. Collins. 1976. Factors affecting haploid plant production from in vitro anther cultures of *Nicotiana* species. Crop Sci. 16 : 837-840.
17. Tsay, H. S. 1980. Anther culture and plant breeding. Sci. Agric. 28 (11-12) :379-396
18. Tsay, H. S. 1980. Tobacco plants formation from anther culture as influenced by the mother plants grown under different seasons. Jour. Agric. Res. China 29 (2) : 147-155.

不同氮素量生長下菸草對花葯培養胚狀體形成的影響[1]

蔡 新 聲[2]

摘 要

利用水耕液培養菸草，發現不同的氮素量可對菸草的發育和生理造成極大的影響。本文之目的在找出母體植物的營養狀況對花葯培養胚狀體形成的影響。

以Hoagland 基本鹽類爲基礎的水耕液培養菸草，發現在五種受測氮素濃度中，以15 mM 最適合植物體生長，而胚狀體形成的最佳情況亦爲 15 mM 氮素量生長下之菸草花葯。減低母體植物氮素濃度會降低花葯培養胚狀體形成率。對母體植物小胞子的形成過程做詳細的切片檢查，發現不含氮素量生長下的菸草花葯，其 tapetum 層發育最差，且小胞子之細胞質較不易染色。因此假定有良好發育 tapetum 層及細胞較具染色力的小胞子，可對菸草花葯培養胚狀體形成的過程造成重大的影響。

---

1. 臺灣省農業試驗所 研究報告 第 968 號。
2. 本所農藝系技正。臺灣省 臺中縣 霧峰鄉。